OECD GUIDELINE FOR THE TESTING OF CHEMICALS
DRAFT PROPOSAL FOR A NEW GUIDELINE 455

(21/11/2008)

The Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals

INTRODUCTION

1. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The Transcriptional Activation (TA) assay described in this Test Guideline is a level 2 "*in vitro* assay, providing mechanistic information". The validation study of the Stably Transfected Transactivation Assay (STTA) by the Japanese Chemicals Evaluation and Research Institute using the hERα-HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (2).

2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) and the androgen receptor (AR) (3)(4)(5)(6). They have been proposed for the detection of estrogenic transactivation regulated by the ER (7)(8)(9). The nuclear ERs exist as at least two subtypes, termed α and β, encoded by distinct genes and with different tissue distribution, relative ligand binding affinities and biological functions. Nuclear ERα mediates the classic estrogenic response, therefore models currently being developed to measure ER activation mainly relate to ERα. The aim of this TA assay is to evaluate the ability of a chemical to function as an ERα ligand and activate an agonist response, for screening and prioritisation purposes.

3. Definitions and abbreviations used in this Test Guidelines are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Estrogen agonists act as ligands for ERs, and may activate the transcription of estrogen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting estrogen-regulated systems. This Test Guideline describes an assay that evaluates transcriptional activation mediated by the hERα. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include (i) actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes, (ii) metabolic activation or deactivation of hormones, (iii) distribution of hormones to target tissues, and (iv) clearance of hormones from the body. This Test Guideline exclusively addresses transcriptional activation of a estrogen-regulated reporter gene by agonist binding to the hERα,

and therefore it should not be directly extrapolated to the complex *in vivo* situation of estrogen regulation of cellular processes. Furthermore, this Test Guideline does not address antagonist interaction with the hERα and subsequent effect on transcription.

5. This test method is specifically designed to detect hERα-mediated transcriptional activation by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over-activation of the luciferase reporter gene (10)(11). While the dose response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (Annex 2).

6. It is recognized that this assay using the hERα-HeLa-9903 cell line is only one of several ER transcriptional activation assays currently being developed and validated. It is, therefore the intention that a generic performance based Test Guideline will replace this Test Guideline as soon as such guideline is developed and approved.

7. This Test Guideline does not address antagonist interaction with the hERα and subsequent effect on transcription; therefore, from the results of this assay it is only possible to determine whether or not a chemical is an ERα agonist. Chemicals, for which test results are negative in this assay, should be evaluated in a receptor binding assay or other assay known to detect antagonists to the ER receptor before the conclusion that the chemical does not bind to the receptor is accepted.

PRINCIPLE OF THE TEST

8. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal binding of the estrogen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

9. The test system provided in this guideline utilises the hERα-HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hERα expression construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently this hERα-HeLa-9903 cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression.

10. Data interpretation for this assay is based upon whether or not the maximum luciferase response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control (PC) 17β

estradiol (E2) (*i.e.*, the PC10). Data analysis and interpretation are discussed in greater detail in paragraphs 36- 46.

PROCEDURE

Cell Lines

11. The stably transfected hERα-HeLa-9903 cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank[1].

12. Only cells characterised as mycoplasma-free should be used in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasm infection.

Stability of the cell line

13. To monitor the stability of the cell line, E2, 17α-estradiol, 17α-methyltestosterone, and corticosterone should be used as the reference chemicals and a complete concentration response curve in the test concentration range provided in Table 1 should be measured in at least one run each day the assay is performed, and the results should be compared with the historical data generated by each laboratory.

Cell Culture and Plating Conditions

14. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of antibiotic Kanamycine and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37±1˚C. Upon reaching 75-90% confluency, cells can be subcultured at 10 mL of $0.4 \times 10^5 – 1 \times 10^5$ cells/mL for 100 mm cell culture dish. Cells should be suspended with 10% FBS-EMEM (which is the same as EMEM with DCC-FBS) and then plated into wells of a microplate at a density of 1×10^4 cells/100 µL/well. Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37˚±1˚C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.

15. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the hERα-HeLa-9903 cell line, this will be less than three months.

16. The preparation of DCC-FBS is described in Annex 3.

Acceptability Criteria

Positive and Negative Reference Chemicals

17. Prior to and during the study, the responsiveness of the test system should be verified

[1] JCRB Cell Bank : National Institute of Biomedical Innovation
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using the appropriate concentrations of a strong estrogen: E2 (CAS No. 50-28-2), a weak estrogen (17α-estradiol; CAS No.57-91-0), a very weak agonist (17α-methyltestosterone; CAS No. 58-18-4) and a negative compound (corticosterone; CAS No. 50-22-6). Acceptable range values from the validation study are given in Table 1. These concurrent reference chemicals should be included with each experiment and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the criteria should be determined (*e.g.,* cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once acceptable criteria have been achieved, to ensure minimum variability of EC50, PC50 and PC10 values, consistent use of materials for cell culturing is essential. The four concurrent reference chemicals, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the PC10s of the three positive reference chemicals should fall within the acceptable range, and the PC50s and EC50s where they can be calculated (see Table 1).

Table 1. Acceptable range values of reference chemicals for the STTA assay (means ± 2 standard deviations).

Name	logPC50	logPC10	logEC50	Hill slope	Test range
17β-Estradiol (E2) CAS No: 50-28-2	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8} M
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6} M
Corticosterone CAS No: 50-22-6	–	–	–	–	10^{-10} ~ 10^{-4}M
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	10^{-11} ~ 10^{-5} M

Note: data is derived from the regression of normalised luciferase activity against the log concentration of reference chemical.

Positive and Vehicle Controls

18. The positive control (PC) (1 nM of E2) should be tested at least in triplicate in each plate. The vehicle that is used to dissolve a test chemical should be tested as a vehicle control (VC) at least in triplicate in each plate. In addition to this vehicle control, if the PC uses a different vehicle than the test chemical, another vehicle control should be tested at least in triplicate on the same plate with the PC.

Fold-induction

19. The mean luciferase activity of the positive controls (1 nM E2) should be at least 4-fold that of the mean vehicle control on each plate. This criterion is established based on the reliability of the endpoint values from the validation study (historically between four- and 30-fold).

20. With respect to the quality control of the assay, the fold-induction corresponding to the PC10 value of the concurrent PC (1 nM E2) should be greater than 1+2SD (standard deviations) of the fold-induction value (=1) of the concurrent VC (vehicle control). For prioritisation purposes, the PC10 value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritisation purposes.

Chemicals to Demonstrate Laboratory Proficiency

21. Prior to testing unknown chemicals in the TA assay, the responsiveness of the test system should be confirmed by each laboratory, at least once for each newly prepared batch of cell stocks taken from the frozen stock, by independent testing of the proficiency chemicals listed in Table 2. This should be done at least in duplicate, on different days.

Table 2. List of Proficiency Chemicals

Compound	CAS No.	Class[2]	Test concentration range	Note
Diethylstilbestrol (DES)	56-53-1	Positive	10^{-14} - 10^{-8} M	
17α-Ethynyl estradiol (EE)	57-63-6	Positive	10^{-14} - 10^{-8} M	
Hexestrol	84-16-2	Positive	10^{-13} - 10^{-7} M	
Genistein	446-72-0	Positive	10^{-12} – 10^{-5} M	Cytotoxic at (0.01)[4], 0.1 and 1 mM
Estrone	53-16-7	Positive	10^{-12} - 10^{-6} M	
Butyl paraben	94-26-8	Positive	10^{-11} – 10^{-4} M	Cytotoxic at (0.1)[4] and 1 mM
4-*n*-Nonylphenol	104-40-5	Positive	10^{-12} – 10^{-5} M	Cytotoxic at 0.1 and 1 mM
1,3,5-Tris(4hydroxyphenyl)benzene[1]	15797-52-1	Positive	10^{-12} - 10^{-5} M	Cytotoxic at 100 μM. PCmax approx 15% of PC Binds to hERα and has ER antagonist activity
Dibutyl phthalate (DBP)	84-74-2	Negative[3]	10^{-11} – 10^{-4} M	Cytotoxic at 1 mM
Atrazine	1912-24-9	Negative	10^{-11} – 10^{-4} M	Cytotoxic [4] at 1 mM
Corticosterone	50-22-6	Negative	10^{-10} – 10^{-4} M	If not cytotoxic at 1 mM, then that should be the highest tested concentration

[1] Compound selected to challenge solubility and cytotoxicity.
[2] See Table 5 for definitions of positive and negative.
[3] Negative for ERα mediated transcriptional activation but may not be negative for non ERα mediated transcriptional activation. Thus a positive result in this assay with DBP would indicate that the system is detecting other than pure ERα mediated activity and is therefore unacceptable.
[4] Cytotoxicity is close to 80%.

Vehicle

22. Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals should be used as the concurrent vehicle control. Test substances should be dissolved in a solvent that solubilizes that test substance and is miscible with the cell medium. Water, ethanol (95% to 100%) and DMSO are suitable vehicles. However DMSO is the preferred vehicle due to lower volatility and greater solubility properties. If DMSO is used, the level should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance.

Preparation of Test Chemicals

23. Generally, the test chemicals should be dissolved in DMSO or other suitable solvent, and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media.

Solubility and Cytotoxicity: Considerations for Range Finding.

24. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any solubility and cytotoxicity problems. Initially, chemicals are tested up to the maximum concentration of 5 μl/ml, 5 mg/ml, or 1 mM, whichever is the lowest. The first run of the assay should be based upon the concentration response curve, with serial concentrations of 1 mM, 100 μM, 10 μM, etc and the presence of cloudiness or precipitate noted. Concentrations in the second, and if necessary third run should be adjusted as appropriate to avoid levels at which the chemical may be found to be insoluble.

25. For ER agonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. If cytotoxicity testing is deemed necessary, cytotoxicity testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience.

26. Should the results of the cytotoxicity test show that the concentration of the test substance has reduced the cell number by 20% or more, this concentration is regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

Chemical Exposure and Assay Plate Organisation

27. The procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-3}-10^{-11} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 1.5 µL of the test chemical in the solvent to a concentration of 500 µL of media.

Step-3: Chemical exposure of the cells: Add 50 µL of dilution with media (prepared in Step-2) to an assay well containing 10^4 cells/100 µL/well.

The recommended final volume of media required for each well is 150 µL.

Test samples and reference chemicals can be assigned as shown in Table 3.

Table 3.: Example of plate concentration assignment of the reference chemicals in the assay plate

Row	**17α-Methyltestosterone**			**Corticosterone**			**17α-Estradiol**			**E2**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	conc 1 (10 µM)	→	→	100 µM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	10 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	1 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (1 nM E2)

28. The reference chemicals (E2, 17α-Estradiol, 17α-methyl testosterone and corticosterone) should be tested in every run (Table 3). Positive control wells (PC) treated with 1 nM of E2 that can produce maximum induction and vehicle control wells (VC) treated with DMSO (or appropriate solvent) alone should be included in each test assay plate (Table 4). If cells from different sources (e.g., different passage number, different lot, etc.) are used in the same experiment, the reference chemicals should be tested for each cell source.

Table 4.: Example of plate concentration assignment of test and plate control chemicals in the assay plate

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 μM)	→	→	1 mM	→	→	1 μM	→	→	10 nM	→	→
B	conc 2 (1 μM)	→	→	100 μM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 μM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 μM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

29. Positive control wells treated with 1 nM of E2 that can produce maximum induction, and vehicle control wells treated with DMSO alone, should be prepared on every assay plate.

30. The lack of edge effects should be confirmed, as appropriate, and if edge effects are suspected, the plate layout should be altered to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

31. After adding the chemicals, the assay plates should be incubated in a 5% CO_2 incubator at 37±1ºC for 20-24 hours to induce the reporter gene products.

32. Special considerations will need to be applied to those compounds that are highly volatile. In such cases, nearby control wells may generate false positives, and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of “plate sealers” may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

33. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

34. A commercial luciferase assay reagent [*e.g.*, Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (Promega, E1500, or equivalents) can be used for the assay, as long as the criteria for the performance standard are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers’ instructions.

ANALYSIS OF DATA

35. To obtain the relative transcriptional activity, the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate mean value for the VC.
Step 2. Subtract the mean value of the VC from each well value to normalise the data.
Step 3. Calculate the mean for the normalised PC.
Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised mean PC (PC=100%).
The final value of each well is the relative transcriptional activity for that well compared to the maximum PC response.
Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

EC50, PC50 and PC10 induction considerations

36. The full concentration response curve is required for the calculation of the EC50, but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC50 and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible. For the calculation of EC50 and maximum induction level, appropriate statistical software should be used (e.g. Graphpad Prism statistical software).

37. If the Hill's logistic equation is applicable to the concentration response data, the EC50 should be calculated by the following equation (12):

Y=Bottom + (Top-Bottom) / (1+10 exp ((Log EC50 -X) x Hill-Slope))
Where:
X is the logarithm of concentration; and,
Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.
Bottom is fixed at zero in the Hill's logistic equation.

38. For each test chemical, the following should be provided:

(i) The RPCMax which is the maximum level of luciferase induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate, as well as the PCMax (concentration associated with the RPCMax); and

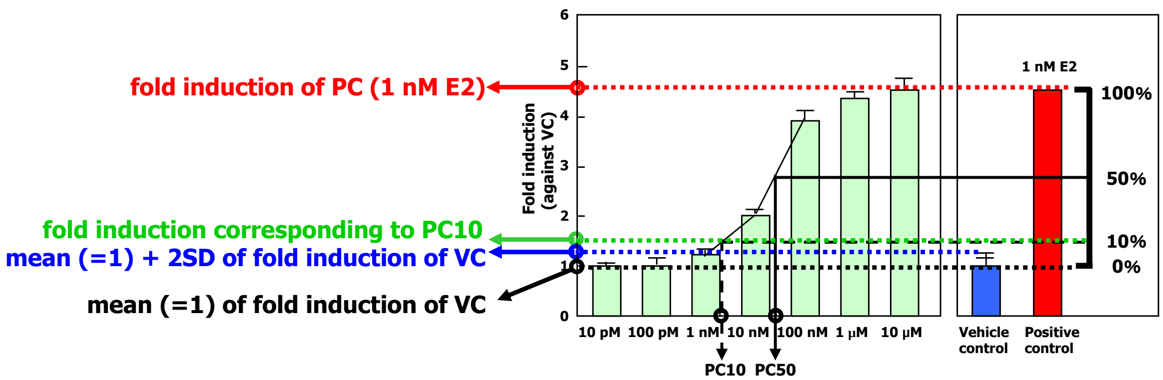
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

39. The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then the PCx value may be calculated using the following equation:

log[PCx] = log[c]+(x-d)/(d-b)

40. Descriptions of PC values are provided in Figure 1 below.

Figure 1.: Example of how to derive PC-values. The PC (Positive control; 1 nM of E2) is included on each assay plate



41. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the performance standard requirements;
  - The mean luciferase activity of the positive controls (1 nM E2) should be at least 4-fold that of the mean vehicle control on each plate
  - The fold induction corresponding to the PC10 value of the concurrent PC (1 Nm E2) should be greater than 1+2SD of the fold induction value (=1) of the VC (vehicle control).
  - The results of reference chemicals should be within the acceptable range (Table 1).
- Be reproducible.

Data Interpretation Criteria

Table 5. : Positive and negative decision criteria

Positive	If the RPCMax is obtained that is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs.
Negative	If the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

42. Data interpretation criteria are shown in Table 5. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC50) or 10% (PC10) of positive control values are reached accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induced by the test chemical (RPCMax) is equal to or exceeds 10% of

the response of the positive control in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

43. The calculations of PC10, PC50 and PCMax can be made by using a spreadsheet available with the Test Guideline on the OECD public website.

44. It should be sufficient to obtain PC10 or PC50 values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (i.e. luminescence intensity data) of the data points that are used for the calculation of PC10 should be less than 20%.

45. Meeting the performance standards indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best insurance that accurate data were produced, see paragraphs 41 and 42.

46. Where more information is required in addition to the screening and prioritisation purposes of this TG for positive test compounds, particularly for PC10-PC49 chemicals, as well as chemicals suspected to over stimulate luciferase, it can be confirmed that the observed luciferase-activity is solely an ERα-specific response, using an ERα antagonist (see Annex 3).

TEST REPORT

47 The test report should include the following information:

Test substance:

- identification data and CAS N°, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle, if known.

Cells:

- type and source of cells;
- number of cell passages;
- methods for maintenance of cell cultures.

Test conditions:

cytotoxicity data and solubility limitations should be reported, as well as:

- composition of media, CO_2 concentration;
- concentration of test chemical;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- Luciferase assay reagents (Product name, supplier and lot);
- criteria for considering tests as positive, negative or equivocal.

Reliability check:

- Fold inductions for each assay plate.
- Actual logEC50, logPC50, logPC10 and Hillslope values for concurrent reference chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- RPCMax, PMax, PC50 and/or PC10 values, as appropriate;
- EC50 values, if appropriate;
- Statistical analyses, if any, together with a measure of error (e.g., SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of the results

Conclusion

LITERATURE

1. OECD (2002). OECD conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [Annex 2 to TG 440]
2. CERI (2006). Draft validation report of TA assay using HeLa-hER-9903 to detect estrogenic activity. [Available at: http://www.oecd.org/document/62/0,3343,en_2649_34377_2348606_1_1_1_1,00.html]
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ANNEX 1

DEFINITIONS AND ABBREVIATIONS

Agonist: A substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand that binds to the same receptor.

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-estrogenic activity, the capability of a chemical to suppress the action of 17β-estradiol mediated through estrogen receptors.

AR: Androgen receptor.

Cytotoxicity: the harmful effects to cell structure or function ultimately causing cell death and can be a result of a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function <u>when compared to the concurrent vehicle</u> control.

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

EC50 value, the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

EE: 17α-ethynyl estradiol

ER; Estrogen receptor

ERE: Estrogen Response Element

Estrogenic activity, the capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors. hERα mediated specific estrogenic activity can be detected in this Test Guideline.

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

MT: Metallothionein

Negative, a chemical that does not induce at least a PC10 in at least two replicate experiments

OHT: 4-Hydroxytamoxifen

PC: Positive control

PC10: the concentration of a test chemical at which the measured activity in an agonist assay is 10% of the maximum activity induced by positive control (E2 at 1nM) in each plate

PC50: the concentration of a test chemical at which the measured activity in an agonist assay is 50% of the maximum activity induced by positive control (E2 at 1nM) in each plate

PCMax: the concentration of a test chemical inducing the RPCMax

RPCMax: maximum level of luciferase activity induced by a test chemical, expressed as a percentage of the response induced by 1Nm E2 on the same plate

RT PCR: Real Time polymerase chain reaction

SD: Standard deviation

STTA: Stably Transfected Transcriptional Activation Assay.

TA: Transcriptional activation

Validation, a process based on scientifically sound principles by which the reliability and relevance of a particular test, approach, method, or process are established for a specific purpose. Reliability is defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol. The relevance of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate (13).

VC: The vehicle that is used to dissolve test and control chemicals is tested solely as vehicle without dissolved chemical.

ANNEX 2

False positives: Assessment of non-receptor mediated luminescence signals

1. False positives might be generated by non-ER-mediated activation of the luciferase gene, or direct activation of the gene product or unrelated fluorescence. Such effects are indicated by an incomplete or unusual dose-response curve. If such effects are suspected, the effect of an ER antagonist (e.g. 4-hydroxytamoxifen (OHT) at 10 µM) on the response should be examined. The pure antagonist ICI 128780 may not be suitable for this purpose as a sufficient concentration of ICI 128780 may decrease the vehicle control value, and this will affect the data analysis.

2. To ensure validity of this approach, the following would need to be tested in the same plate:

- Agonistic activity of the unknown chemical with / without 10 µM of OHT
- Vehicle Control (VC)
- 10 µM of OHT (in triplicate)
- 1 nM of E2 (in triplicate) as agonist Positive Control (PC)
- 1 nM of E2 + 10 µM of OHT (in triplicate)

3. ***Data interpretation***

Note: All wells must be treated with the same concentration of the vehicle.

- If the agonistic activity of the unknown chemical is NOT affected by the treatment with ER antagonist, it is classified as “Negative”.
- If the agonistic activity of the unknown chemical is completely inhibited, simply apply the decision criteria.
- If the agonistic activity of the unknown chemical is partly inhibited (as below), calculate the difference in the responses between the non-treated and treated wells with the ER antagonist. This difference between the treated and non-treated wells should be considered as the true response and should be used for the calculation of the appropriate parameters to enable a classification decision to be made.

4. ***Data analysis***

Check the performance standard.

Check the CV between wells treated under the same conditions.

1. Calculate the mean of the VC
2. Subtract the mean of VC from each well value **not** treated with 10 µM of OHT
3. Calculate the mean of 10 µM of OHT
4. Subtract the mean of the VC from each well value treated with 10 µM of OHT
5. Calculate the mean of the PC
6. Calculate the relative transcriptional activity of all other wells relative to the PC.

ANNEX 3

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

1. The treatment of serum with dextran-coated charcoal (DCC) is a general method for removal of estrogenic compounds from serum that is added to cell medium, in order to exclude the biased response associated with residual estrogens in serum.

Components

2. The following materials and equipment will be required:

Materials

Activated charcoal
Dextran
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Sucrose
1 M HEPES buffer solution (pH 7.4)
Ultrapure water produced from a filter system

Equipment

Autoclaved glass container (size should be adjusted as appropriate)
General Laboratory Centrifuge (that can set temperature at 4°C.)

Procedure

3. The following procedure is adjusted for the use of 50 mL centrifuge tubes:

[Day-1] Prepare dextran- coated charcoal suspension with 1 litre of ultrapure water containing 1.5 mM of $MgCl_2$, 0.25 M sucrose, 2.5 g of charcoal, 0.25 g dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10.000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with fetal bovine serum (FBS) that has been gently thawed to avoid precipitation, and heat-inactivated at 56°C for 30 minutes, then transfer into an autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10.000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10.000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.2µm sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.